Separation of splenic red and white pulp occurs before birth in a LTαβ independent manner

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Abstract

For the formation of lymph nodes and Peyer’s patches, lymphoid tissue inducer cells are crucial in triggering stromal cells to recruit and retain hematopoietic cells. Although LTi cells have been observed in fetal spleen, not much is known about fetal spleen development and the role of LTi cells in this process. Here we show that LTi cells collect in a periarfetioriolar manner in fetal spleen at the periphery of the white pulp anlagen. Expression of the homeostatic chemokines can be detected in stromal and endothelial cells, suggesting that LTi cells are attracted by these chemokines. Since LTα1β2 can be detected on B cells, but not LTi cells, in neonatal spleen starting at 4 days after birth, the earliest formation of the white pulp in fetal spleen occurs in a LTα1β2 independent fashion. The postnatal development of the splenic white pulp, involving the influx of T cells, depends on LTα1β2 expressed by B cells.
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Introduction

The spleen is composed of a branching splenic artery that eventually ends in venous sinuses. The arterial branches, central arterioles, are surrounded by a layer of lymphoid tissue, the white pulp. It consists of T cell areas and B cell follicles, more or less resembling the organization found in lymph nodes. The arterial blood ends in a sinusoid system in the area surrounding the T and B cell zones, thereby forming an anatomical border between the white and red pulp, the marginal zone. Here sinusoid spaces formed by lining cells continuous with the endothelium of the arterioles and reticular fibroblasts make up a network through which the blood freely percolates on its way to the red pulp and can be scanned for pathogens and debris by macrophages and dendritic cells. The blood runs from the marginal zone through the red pulp cords into the venous sinuses, enabling the spleen to exert its function as a filter of the blood by removal of dysfunctional red blood cells. For development and organization of lymphoid organs the members of the TNF superfamily are crucial. The organogenesis of Peyer’s patches (PP) and lymph nodes (LN) is dependent on the expression of LTαβ2 and other TNF-family members. During fetal and neonatal life, LTαβ2 is expressed by CD45+CD4+Il7r+CD3- lymphoid tissue inducer (LTi) cells in PP and LN anlagen. LTi cells are attracted by non-hematopoietic stromal cells that express the homeostatic chemokines CCL19, CCL21, and CXCL13 as well as the adhesion molecules VCAM-1, ICAM-1, and MAdCAM-1. These molecules are thought to be induced by the interaction of LTi and stromal cells, which leads to lymphotoxin beta receptor (LTβR) and TNFR-I triggering. Expression of these molecules favors the subsequent recruitment and retention of more LTi cells as well as other hematopoietic cells.

For the proper development of the murine neonatal lymphoid part of the spleen, indications that LTαβ2 is involved so far only stem from data obtained after birth, that show LTαβ2 expressing B cells are required for the induction of sufficient CCL21, produced by stromal cells in the T cell zone areas. Considering the important role of LTi cells in the organogenesis of other lymphoid organs relatively little is known about their role in spleen development, although their presence has been demonstrated in the fetal spleen as early as E13.5. Transfer of in vitro II7 activated splenic LTi cells was shown to restore the B/T segregation in spleens of LTα-/- mice, suggesting a role for LTi cells in white pulp development. Since these experiments may not reflect the actual interactions and cellular requirements during fetal and neonatal spleen development we studied the role of LTi cells in the developing splenic white pulp in more detail. Our results show that neonatal splenic LTi cells lack LTαβ2 expression, and that LTαβ2, required for correct formation of the splenic white pulp, is expressed by B cells starting around 4 days after birth. Analysis of the earliest events during spleen development demonstrates that compartmentalization of red and white pulp areas starts to be regulated during embryogenesis. By grafting fetal spleens, distinct white pulp areas, containing donor derived LTi and B cells and red pulp areas, harbouring erythrocytes, can be observed. Therefore the white pulp stroma is already primed before E15.5 to segregate away from the red pulp areas. Moreover, we suggest that this early phase of stroma instruction is independent of the LTβR pathway during murine spleen development.
Materials and Methods

Animals
C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and from Charles River (Saint Germain sur l'Arbresle, France), lymphotoxin α (LTα)−/− mice were purchased from Charles River (Maastricht, The Netherlands). All mouse strains were bred in the Animal Facilities of the VU University Medical Center or the Pasteur Institute and kept under routine laboratory conditions. All animal procedures were approved by the ethical committees of the VUMC and Pasteur Institute.

Timed pregnancies
Mice were mated overnight, and the day of vaginal-plug detection was marked as embryonic day 0.5 (E0.5). Pregnant females were sacrificed at different time points, and embryos were harvested and either frozen in OCT embedding medium (Sakura Finetek Europe BV., The Netherlands) or used for dissection.

Immunofluorescence
Seven µm cryosections were fixed in dehydrated acetone for 2 min and air-dried for an additional 15 min. Endogenous avidin was blocked with an avidin-biotin block (Vector Laboratories, Burlingame, CA, USA). Sections were then pre-incubated in PBS supplemented with 5% (v/v) mouse serum for 10 min. Incubation with primary Ab for 45 min was followed by a 30 min incubation with Fluor-Alexa-labeled conjugate (Invitrogen Life Technologies, Breda, The Netherlands) when needed. All incubations were carried out at room temperature. Before embedding in polyvinyl alcohol, sections were counterstained with Hoechst 33342 (Invitrogen Life Technologies, The Netherlands) for 10 min. Stainings were analyzed on a Leica TCS-SP2-AOBS Confocal Laser Scanning Microscope (Leica Microsystems Nederland BV, The Netherlands) and images were obtained with Leica confocal software. Image processing involved contrast enhancement and region of interest selection, which was carried out with Jasc Paintshop Pro 7.0. Lenses used were dry-lenses: 20x (HC PL APO CS 0.7), 40x (HCX PLAN APO 0.85).

Fetal spleens grafted under the kidney capsule were dissected and incubated overnight in 15% saccharose PBS at 4°C prior to cryosectioning. Sections were analyzed on a Zeiss Axioplan 2 imaging upright microscope with Zeiss Axiocam Hrc camera and Zeiss Axiovision 4.2 software.

Whole mount fetal spleen Immunostaining
E15.5 fetal spleens were incubated O/N in 4% paraformaldehyde in PBS while agitating at 4°C. Spleens were washed twice in PBTr solution (PBS1X, 10% fetal calf serum, 0.1 % triton X100) while agitating during 90 min. and incubated O/N with RMA4-5 (anti-CD4-FITC) antibody at 4°C under agitation. After washes spleens were stained with Hoechst 33342 for 90 min, washed and then embedded in Vectashield (Vector Laboratories) and analyzed on a Zeiss Axioplan 2 imaging upright microscope with Zeiss Axiocam Hrc camera and Zeiss Axiovision 4.2 software.
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Fetal spleen grafts
The fetal spleen harvested from E14.5 to E16.5 C57BL/6 (CD45.2) embryos were placed under the kidney capsule of Rag2/γc−/− (CD45.1) mice by surgery. The Rag2/γc−/− (CD45.1) recipient mice were anesthetized by peritoneal injection of 1.4 mg/g of ketamine (Merial, France) and 7 µg/g of xylazine (Sigma-Aldrich Steinheim, Germany) diluted in PBS. Two hours before the anesthesia, some Rag2/γc−/− (CD45.1) recipient mice were injected with a total C57BL/6 (CD45.2) fetal liver cell suspension. For negative control, sham operated recipients were anesthetized and their kidney capsule was opened without grafting a fetal spleen.

Antibodies
For flow cytometry and immunofluorescence, the following Abs were used: GK1.5 (anti-CD4), MECA-367 (anti-mucosal addressin cell adhesion molecule-1 (MAdCAM-1)), MP33 (anti-CD45, BD Biosciences, Belgium), 6B2 (anti-B220), 3E2 (anti-ICAM-1) and MOMA-2 (a pan macrophage marker). All the Abs were affinity purified from hybridoma cell culture supernatants with protein G-Sepharose (Pharmacia, Uppsala, Sweden) and biotinylated or labeled with Alexa-Fluor 488, Alexa-Fluor 546 or Alexa-Fluor 633 (Invitrogen Life Technologies). 429 (anti-VCAM-1, BD Biosciences, Belgium), A7R34 (anti-II7r, eBioscience), 11D4.1 (anti-VE-cadherin, BD Biosciences), RAM4-5 (anti-CD4, BD Biosciences), 30-F11 (anti-CD45, BD Biosciences), 104 (anti-CD45.2, BD Biosciences), RA3-6B2 (anti-B220, BD Biosciences), 1D3 (anti-CD19, BD Biosciences), Ter-119 (BD Biosciences), PK136 (anti-NK1.1, BD Biosciences), RAM34 (anti-CD34, BD Biosciences), Avas 12α1 (anti-VEGFR2, BD Biosciences), HL3 (anti-CD11c; BD Biosciences), 145-2C11 (anti-CD3ε; BD Biosciences), 7G6 (anti-CD21, BD Biosciences), and A20 (anti-CD45.1, BD Biosciences) anti-VE-cadherin (Alexis Corporation, Switzerland) were used as biotinylated, fluorescently labeled, or as unconjugated primary antibodies. The antibody anti-rat-IgG-TRITC (Chemicon International, CA) was used as a secondary antibody.

Flow cytometry
Lymph nodes (LNs) and spleens were dissected using a stereomicroscope, and single cell suspensions were made by digestion with 0.5 mg/ml collagenase type IV (Sigma-Aldrich) in PBS, 2% FBS for 30 min at 37°C while constantly stirring. For surface LTα1β2 detection, cells were pretreated with 2.4G2 (anti-CD16/32) supplemented with 5% normal mouse serum for 30 min and subsequently incubated with an LTβR-human IgG fusion protein for 60 min. Anti-human-IgG-PE (Jackson Immunoresearch Laboratories, West Grove, PA) was used as second-step conjugate. Splenocytes from adult LTα1β−/− mice were used as negative controls. Flow cytometric analysis was performed on a FACS Calibur using CellQuest software (BD Biosciences). Cells were negatively gated for TCRαβ to exclude all T-cells. 7-Aminoactinomycin D (Molecular Probes, OR) was used to exclude dead cells. For each staining, negative control stainings were carried out, in which LTβR-human IgG fusion protein was incubated together with anti LTβ mAb (BBF6), which prevents the binding of LTβR-human IgG (Alexis Benelux, The Netherlands) to cell surface LTα1β2.
For analysis of fetal spleen and fetal spleen grafts the organs were dissected using a stereomicroscope and single cell suspensions were made by dissociation with a 26 gauge 3/8 inches needle. Propidium iodide (Sigma-Aldrich) was used to exclude dead cells. Flow cytometric analyses were performed in an upgraded LSR (Becton Dickinson, CA) using FlowJo software (Tree Star Ashland, OR).

RT-PCR, Semi quantitative PCR and quantitative real-time PCR

Stromal cells from E15.5 fetal spleen were sorted by flow cytometry on a MOFLO (Dako, Fort Collins, CO), lysed in RLT buffer (Qiagen, Germany) and frozen on dry ice. Total RNA was extracted with RNeasy Micro Kit (Qiagen) according to the manufacturer’s protocol. The cDNA were prepared from 3x10³ to 3x10⁶ cells using random primers, SuperScript II reverse transcriptase, Rnase OUT in a reaction volume of 24 µl. PCR reactions (Invitogen Life Technologies) were performed with Amplitaq gold (Applied Biosystems, NJ) in a reaction volume of 25 µl.

The primers used were: β-actin, for: CCGCGAGCACAGCTTTT, β-actin, rev: CTTTGACATGCCGGAGC, Vcam-1, for: GCTCTGGGAAGCTGGAGA, Vcam-1, rev: TTCATGAGCTGGTCACCCTTG. cDNA was diluted at 1/10 and 1/100 and results from the 1/100 dilution were used to measure intensity of the band using the ImageJ 1.38 application. Intensity was normalized using the β-actin transcript levels (100%).

The cDNA samples used for quantitative real-time PCR were obtained by using RT² PCR Array First Strand Kit (SuperArray Bioscience Corporation, MD) for the reverse transcription step. The RT² Profiler PCR Array ‘Mouse Chemokines & Receptors’ (SuperArray) and the RT² Real-Time SYBR Green/ROX PCR MasterMix (SuperArray) were used for real-time PCR quantification. The real-time PCR were performed on a 7300 Real Time PCR Systems (Applied Biosystems, NJ). The products of each reaction were checked by 2% agarose gel electrophoresis migration.

CD4⁺Il7r⁺ CD3ε⁻ cells from 3 days old mice intestine previously dissociated by dispase (GibcoBRL) treatment and CD4⁺Lin⁻ cells (Lin: Gr-1, B220, Ter119, CD11c, CD19, NK1.1, CD3ε) cells from E15.5 fetal spleen were sorted by flow cytometry on a MOFLO (Dako, CO), lysed in TRizol (GibcoBRL) and frozen at -20°C. Total RNA was extracted according to the TRizol (GibcoBRL) manufacturer’s protocol. Oligo (dT)-primed cDNA was prepared using SuperScript II reverse transcriptase (Invitogen Life Technologies) in a reaction volume of 20 µl. PCR reactions were performed with Amplitaq gold (Applied Biosystems) in a reaction volume of 25 µl. The primers used were: RORγ/γ(t), for: GCCTCCTGAGACGCCAG, RORγ/γ(t), rev: CACCTCTCCCGTGAAAG.
Characterization of the developing spleen

Human fetal spleen development was recently characterized by Steiniger and coworkers \textsuperscript{27}. They could observe distinct areas where the homeostatic chemokine CXCL13 was expressed already at the end of stage I of spleen development (ranging from the 14th to 21st week of gestation), when the first lymphocytes start to colonize the organ. The presence of CXCL13 in arterial smooth muscle cells as well as cells around these arterioles \textsuperscript{27}, suggested the interaction of these stromal cells with LTi cells, in analogy with the developing lymph nodes, and a role in the further development of the spleen. To further define the earliest events in spleen development we addressed whether also these distinct areas could be found in murine spleen. Hereeto fetal spleens from E18.5 were stained for endothelial, hematopoietic as well as stromal markers. Combined staining of Tie-2/Tek, a marker for endothelial cells \textsuperscript{28}, and CD45 revealed the presence of an artery, with a contracted lumen, and a vein, with an open lumen in between hematopoietic cells at this time point in development (Fig. 1A, D). CD45\textsuperscript{+} hematopoietic cells were remotely located in a ring like pattern around the artery (Fig. 1B) while the venous blood vessel was immediately surrounded by hematopoietic cells (Fig. 1C). To define the expression of MAdCAM-1, a marker for lymph node stromal organizer cells \textsuperscript{29} as well as high endothelial venules (HEVs) \textsuperscript{30}, fetal spleens were stained for MAdCAM-1 in combination with the endothelial marker VE-Cadherin \textsuperscript{31}. The results showed that most VE-Cadherin\textsuperscript{+} endothelial cells expressed MAdCAM-1 at high levels (Fig. 1E and Suppl. Fig 1A-C). Stainings of subsequent sections showed that endothelial cells of large vessels also expressed ICAM-1, while VCAM-1 was either absent or expressed at low levels (Fig. 1F). In addition, around the artery, VCAM-1\textsuperscript{+} cells were found. Since LTi cells were reported by us to be present at early stages in developing spleen \textsuperscript{8}, we performed additional stainings for Il7r and CD4 in subsequent sections to detect these cells. Interestingly, CD4\textsuperscript{+}Il7r\textsuperscript{+} LTi cells were located in a ring-like pattern at the periphery of the VCAM-1\textsuperscript{+} stromal cells, adjacent to MAdCAM-1\textsuperscript{+}VE-Cadherin\textsuperscript{+} endothelial cells (Fig. 1E-G). The distinct locations of these cells types are depicted in a schematic drawing (Fig. 1H). To confirm that CD4\textsuperscript{+}Il7r\textsuperscript{+} cells were indeed LTi cells, we performed triple stainings for RORγ(t), which is indispensable for LTi differentiation \textsuperscript{32}, II7r and CD4. These stainings showed that all CD4\textsuperscript{+}Il7r\textsuperscript{+} contained RORγ(t), indicating that these cells were indeed LTi cells (Suppl. Fig. 1D-H). Very few RORγ(t)\textsuperscript{+} II7r\textsuperscript{+}CD4\textsuperscript{+} cells were found at developmental stage E18.5 (data not shown).

To find out whether a distinct pattern of LTi cells in the proximity of vessels could also be observed at earlier stages of spleen development, serial sections of E14.5 and E16.5 spleens were analyzed. Therefore, we investigated the vasculature of the fetal spleen in more depth, by staining sections of E16.5 and E14.5 fetal spleens for VE-Cadherin combined with DAPI and MECA32 staining. The latter antibody has been described as an endothelial marker \textsuperscript{33}. The combination of both endothelial markers allowed us to focus on the artery, which can be distinguished
by expression of VE-Cadherin and lack of MECA32 (VE-Cadherin^MECA32^-). At E16.5 in development we could identify LTi cells around this VE-Cadherin^MECA32^- artery, again organized in a ring-like pattern. In addition, we found single LTi cells and small poorly organized clusters in other areas of the fetal spleen (Fig. 2A-D). At E16.5, most of the vessels were MAdCAM-1^+ (Suppl. Fig. 1I-K), similar to E18.5 splenic vessels. In addition, VCAM-1^+ stromal cells could be distinguished that were organized around the large VE-Cadherin^MECA32^- artery (Fig. 2B, C). Stromal cells and LTi cells appeared to be intermingled, instead of strictly separated as seen in E18.5 fetal spleens. Although at E14.5 LTi cells could be detected in the fetal spleen in the vicinity of blood vessels, they were not yet organized in a ring-like structure as observed at E16.5 and E18.5 (Fig. 2E-H). Strikingly, we could only identify one major blood vessel in the center of the spleen at E14.5. The lumen of this vessel was large, and apart from the expression of VE-Cadherin, it was weakly stained for MECA32 (Fig 2F). In addition, at this time point in development, numerous smaller vessels expressed MAdCAM-1 and ICAM-1, but VCAM-1 expression in the fetal spleen was limited at this developmental time point (Fig 2G and Suppl. Fig. 1L-O). VCAM-1, expression could be observed in deeper layers of the stomach wall, indicating that it was not due to a detection failure (Suppl. Fig. 1L, M). To see whether LTi cells indeed preferentially distribute to certain splenic areas, whole mount E15.5 spleens were stained for CD4. These stainings showed that CD4 cells were clustered, and that these clusters were distributed linearly along the length of the entire spleen (Fig. 2I), indicating that the early compartmentalization of the fetal spleen may attract LTi cells to periarteriolar domains. In addition, a limited number of single CD4^+ cells were found in the spleen at other areas.

**Distinct stromal subsets are present in the fetal spleen**

As LTi cells are localized within special areas of the spleen, we assumed that these cells are co-localizing with a specific stromal cell subset and that various stromal cell populations could be expected to exist in fetal spleens. We therefore analyzed stromal cells present in E15.5 fetal spleen by FACS (Fig. 3A). After elimination of leucocytes (CD45^+ ) and erythrocytes (Ter119^+ ), the E15.5 fetal spleen stroma could be separated into two populations, based on the expression of VE-Cadherin. As can be inferred from immunofluorescent stainings (Fig. 1, 2), splenic stromal cells that surround arterial vessels lack endothelial markers such as VE-Cadherin. In addition, endothelial cells express VE-Cadherin and are CD45^- determined by FACS analysis (Fig. 3A, B). To confirm that VE-Cadherin^+ cells indeed represent endothelial cells, expression of other endothelial markers was analyzed. While 17% of the CD45-Ter119^- cells could be distinguished as a population of CD34^+ICAM1^+VEGFR2^+VE-Cadherin^+Tie-2^- endothelial cells, the remaining 82% of fetal spleen CD45^+ Ter119^- cells were characterized as CD34^-ICAM1^-/lowVEGFR2^+VE-Cadherin^-Tie-2^- stromal cells (Fig. 3A, B). By semiquantitative RT-PCR, we found VCAM-1 transcripts in both subsets with a lowest quantity in the CD34^- stromal subset (Fig. 3C). Since LTi cells were found to be associated with endothelial cells in the spleen, we addressed whether splenic CD45^- cells could produce the chemokines that are known to attract LTi cells. Quantities of CXCL12, CXCL13, CCL19, and CCL21b transcripts were determined in CD34^- stromal and CD34^- endothelial
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**Figure 1:** Murine fetal spleens at developmental stage E18.5. Immunofluorescence stainings (1A-C, E-G) and schematic drawings (1D, H). 1E-G depict serial sections. (A) Murine fetal spleen (FS) consisting of Tie-2+ endothelial cells and CD45+ hematopoietic cells (Tie-2 in green, CD45 in red). The outline of the FS is indicated in white. A major vein (1A, C; arrowhead) and artery (1A, B; double arrowhead) can be distinguished at this time point in development. (D) Schematic drawing of FS showing outline and position of vein and artery. (E) High magnification image of blood vessel endothelial cells expressing MAdCAM-1 (in green) and VE-cadherin (in red). VE-cadherin+ blood vessels with highest MAdCAM-1 expression (yellow) are marked by arrows. Vein (arrowhead) and artery (double arrowhead) are MAdCAM-1+VE-cadherin+ (orange). (F) Expression of ICAM-1 (in red) and VCAM-1 (in green) by endothelial cells and splenic stromal cells. ICAM-1+VCAM-1+ arterial endothelial cells (orange) are immediately surrounded by VCAM-1+ stromal cells (double arrowhead; green). The outline of these cells is indicated in white. (G) LTi cells expressing Il7r (in green) and CD4 (in red) positioned distal to the artery and VCAM-1+ stromal cells, forming a ring-like structure (double arrowhead) that is not found around the vein (arrowhead). Schematic drawing of the outlines of arterial endothelial cells (i), VCAM-1+ICAM-1- stromal cells (ii) and LTi cells forming a ring-like structure (iii). Outward from this structure, blood vessels with highest MAdCAM-1 expression are found. Overview image of fetal spleens are shown at 20x magnification in A. Detailed images of fetal spleens are shown at 40x magnification, zoom 2 in B, C, E-G. Data are representative of 4 individual mice.
subsets of CD45⁻Ter119⁻ cells (Fig. 3D). The expression levels of CXCL12 and CXCL13 were relatively similar in both CD45⁻ Ter119⁻ subsets. In contrast, levels of CCL19 and CCL21b transcript expression were 100 and 1000 fold higher in the CD34⁺ endothelial fraction than in the CD34⁻ stromal fraction, respectively.

White pulp anlagen is already primed in fetal spleen
The expression of homeostatic chemokines by CD45⁻Ter119⁻ cells and the preferential localization of LTi cells to VE-Cadherin⁺ endothelial cells at the periphery of the MAAdCAM-1⁺ stromal areas raised the question whether these periarteriolar areas constituted the anlagen of the white pulp. To address whether these designated areas could mature into splenic white pulp areas, spleens from E15.5 C57BL6 embryos were grafted under the kidney capsule of adult Ly5.1 Rag2/γc⁻/⁻ immunodeficient mice (Fig. 4A). The use of Rag2/γc⁻/⁻ alymphoid mice was essential to avoid substantial contamination by host-derived lymphocytes. B cell precursors are present in fetal spleen at the time of transplantation and they are able to differentiate into mature CD19⁺B220⁺ B cells in situ. When the grafts were analyzed at three weeks after transplantation they showed good vascularization and had increased in size, showing that the hematopoietic progenitors present in E15.5 fetal spleens were able to proliferate and differentiate in situ. Grafts performed with E14.5 spleen also showed proliferation and differentiation of CD45⁺ progenitors within the grafted spleens (data not shown).

Analysis by immunofluorescence showed that B cells and LTi cells co-localized in an area that did not contain Ter119⁺ erythrocytes, indicative of a B/LTi containing white pulp area and a Ter-119 containing red pulp area (Fig. 4A). In Figure 4A, only LTi cells are stained in order to clearly observe the structure of the spleen (LTi clusters and vein). Development of these areas within the grafted spleens indicated that the stroma had been instructed to retain LTi cells to restricted areas that are common to B cells. It appeared that the designation of the white pulp anlagen, containing LTi cells, had already taken place at E15.5, independently of mature circulating lymphocytes.

In parallel, splenic grafts were analyzed by FACS to assess the presence of donor derived LTi, B, NK and T cells (Fig. 4B). Depending on the graft, the proportion of hematopoietic donor cells (CD45.2⁺) varied, whereas the percent of host (CD45.1⁺) hematopoietic cells, containing myeloid and CD11c⁺ dendritic cells, was constant (around 10%) (Fig. 4B). Within the pool of donor-derived hematopoietic cells, clear populations of LTi (CD4⁺CD3⁻Il7r⁺) and B (CD19⁺B220⁺) cells were seen, whereas some of these B cells expressed high levels of CD21, potentially representing MZ B cells (Fig. 4B). Few graft derived NK cells could be observed (Fig. 4B).

Injection of E15.5 total fetal liver cells from C57BL/6 embryos during splenic transplant permits to fully reconstitute the hematopoietic compartment of the alymphoid recipients. In these cases, the B cells, T cells and erythrocytes were clearly distributed into organized white and red pulp areas. The white pulp areas showed normal T/B segregation, indicating that T and B cells could normally enter the white pulp areas (Fig. 4A).

LTα₁β₂ expression during spleen development
Figure 2: Murine FSs at stages E14.5, E15.5 and E16.5 of development. Immunofluorescence staining of serial sections from E16.5 (A-D) and E14.5 (E-H) spleens. (A) Double staining for MECA32 (in green) and VE-cadherin (in red) counterstained with DAPI (in blue) to visualize nuclei. Most of the smaller vascular blood vessels appeared to express both MECA32 and VE-cadherin. (B) Higher magnification image showing a MECA32+ vein that is VE-cadherin− (arrowhead) and a VE-cadherin+ artery that lacks expression of MECA32 (double arrowhead). (C) ICAM-1+VCAM-1+ artery (double arrowhead) surrounded by VCAM-1+ stromal cells (ICAM-1 in red, VCAM-1 in green). (D) Il7r+CD4+CD45lo LTi cells are organized around arterial endothelial cells (CD45 in green, Il7r in red, CD4 in blue). (E) E14.5 spleen (arrow) and stomach (arrowhead) containing MECA32+VE-cadherin+ blood vessels (MECA32 in green, VE-cadherin in red) counterstained with DAPI (in blue). (F) At E14.5 all blood vessels (orange) are double positive for MECA32 and VE-cadherin including a larger blood vessel. The outline of the larger vessel is indicated in white. (G) At this time point, most endothelial cells express MAdCAM-1 (in blue) and ICAM-1 (in red), while VCAM-1 expression is virtually absent in the FS (in green). (H) In the near vicinity of the larger vessel, CD45+ hematopoietic cells are localized, including Il7r+CD4+CD45lo LTi cells (CD45 in green, ll7r in red, CD4 in blue). Images showing sagittal sectioned FS (E16.5) or transverse sectioned FS and stomach (E14.5) at 20x magnification (a, e). Higher magnification images of fetal spleens are shown at 40x and zoom 2 in B-D, F-H. The outline of major blood vessels is marked in white. (I) On E15.5 FS, whole mount immunofluorescent staining was performed to locate CD4+ cells. Data are representative of 4 (E14.5) or 5 (E15.5, E16.5) individual mice.
LTi cells in developing lymph nodes are instrumental for the induction of homeostatic chemokines in developing lymph nodes through their expression of LTα1β2. According to the literature, the development of the spleen requires LTα1β2 expressing cells postnatally, whereas we showed here that homeostatic chemokines could already be observed before birth. Therefore, we addressed at what time point the earliest LTα1β2 expressing cells could be observed, using FACS analysis of postnatal spleens. Mesenteric lymph nodes (MLN) from the same animals were included as positive controls. Analysis of day 0, day 2, day 4, and adult wild type spleens revealed a gradual increase of LTα1β2 on splenic B cells (Fig. 5A). At day 0 and day 2, only 1% of all B cells expressed LTα1β2, while this increased to 2.7% at day 4. In adult spleens, 12.5% of total B cells expressed LTα1β2 (Fig. 5B).

In contrast, few LTα1β2 expressing LTi cells could be detected in neonatal spleens, while LTi cells from MLN expressed high levels of LTα1β2, in accordance with our earlier report (Fig. 5C). When compensated for aspecific binding (Suppl. Fig. 3), the percentage of LTα1β2+ LTi cells in the spleen varies between 1.5% to 2.2% of all LTi cells implicating that LTα1β2+ LTi cells are very rare (Fig. 5D).

**LTα1β2 dependent white pulp development**

As we observed very few LTα1β2 expressing cells at day 2 after birth, we reasoned that before day 2 splenic development, including the formation of the white pulp anlagen, occurred independently of LTα1β2 mediated LTβR triggering. To confirm this, spleens from LTα-/- mice taken at day 2, 4, and 6 after birth were analyzed. In both wild type and LTα-/- mice, small accumulations of B220+ B cells could be found at 2 days after birth, around arterioles in the spleen, indicative of undisturbed formation of white pulp areas. In addition, very few CD3+ T-cells were seen at this time point in or near B cell areas in both wild type and LTα-/- animals (Fig. 6A). The effect of the absence of LTα1β2 expressing cells could be observed around day 4, when B cell areas were still small in LTα-/-, while they had clearly increased in size in WT mice from day 2 to day 4. In addition, T cell areas were completely absent in LTα-/- spleens at all stages analyzed, while these were clearly present in WT spleen at day 4, and had further enlarged at day 6 (Fig. 6A). Despite the dramatic differences for the B and T cell population, we could not find a clear difference with respect to MOMA-2+ macrophages. In fact, the amount of MOMA-2+ cells that were found in close proximity to central arterioles appeared even higher in LTα-/- spleens when compared to wild type spleens at day 4 (Fig. 6A and data not shown).

An additional difference was observed when splenic white pulp stromal cell populations of wild type mice were compared with LTα-/- mice. When staining for VE-cadherin, VCAM-1, and ICAM-1, VE-cadherin negative stromal cell populations could be analyzed. VCAM-1+ICAM-1lo (V+Ilo) cells were located directly around the central arteriole. At the periphery of this white pulp area VCAM-1+ICAM-1+ cells could be seen. When WT and LTα-/- spleens were compared, these cellular subsets could be distinguished at day 2, but the increase in VCAM-1+ICAM-1+ at the periphery of the white pulp area seen at day 4 and day 6 in WT, was not observed in LTα-/- mice (Fig. 6B). These results indicate a LTα1β2+ mediated increase of VCAM-1+ICAM-1+ at the location where the marginal zone will develop, and is in
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Figure 3: The stromal compartment of E15.5 fetal spleen. (A) At E15.5, the fetal spleen stroma was isolated as CD45 − PI − Ter119 − cells and two subsets were distinguished based on CD34 and ICAM1 expression (PI: propidium iodide). Most cells were CD34− ICAM1−/lo and a small subset was formed by CD34+ ICAM1+ cells. (B) These subsets of stromal cells were analyzed for the surface expression of VEGFR2, VE-cadherin and Tie-2. (C) Semi-quantitative RT-PCR analysis of the Vcam1 gene in the E15.5 fetal spleen (FS) populations (dilution with a factor 10). Intensity was quantified to measure the difference of Vcam1 expression normalized by β-actin levels. (D) Quantitative real time PCR were performed to study the relative expression of Cxcl12, Cxcl13, Ccl19 and Ccl21b chemokines from the CD34− ICAM1− (blue histograms) and CD34+ ICAM1+ (orange histograms) stromal populations. The average of Gadph and Hprt housekeeping gene levels were used for the normalization. Results are obtained from two independent cell sorting and each sorted population is represented by a specific histogram.
agreement with earlier observations that LTαβ/LTβR interactions are mandatory for homeostasis and proper functioning of marginal zones in adult spleens. 16, 36-39.

Discussion

The potential role of LTi cells in white pulp ontogeny has been a matter of debate since these cells express the ligand for the LTβR in developing lymph nodes and signaling through this receptor is indispensable for normal white pulp development. 37, 40. Although it has been shown that triggering of the LTβR by LTαβ-expressing B cells is crucial for white pulp development, the possibility remained that triggering of the LTβR by other cells, such as activated T cells or LTi cells, further facilitated this process. Here we show for the first time that the majority of splenic LTi cells lack cell surface expression of LTβR ligand. It has been shown that fetal and neonatal splenic LTi cells express the LTα and LTβ transcripts. 9, 41. The adult spleen environment, but not the fetal, has also been proposed to regulate the LTβ transcript expression in LTi cells by TL1A 41. Our results suggest that the neonatal splenic environment does not lead to cell surface expression of LTβR ligands on LTi cells. An in vitro dose-dependent Il7 stimulation is required for an effective LTβR ligand expression by intestinal LTi cells. 10. We previously detected Il7 transcript expression by fetal spleen non-hematopoietic cells. 42. At day 4 after birth, the time point that T cell areas start to emerge, a ligand for the LTβR, most likely LTαβ2, is only expressed by B cells. Thus for white pulp development B cells will give the LTαβ dependent inductive signal, similar as LTi cells do during LN and PP formation and this is in accordance with earlier observations. 14. This signal is required to attract more B and T cells to the developing white pulp. In contrast to the white pulp development into functional T and B cell areas, the separation of white and red pulp areas occurs before birth and is independent of LTα expressing cells.

In E15.5 fetal spleen, patches of LTi cells are found throughout the spleen with a distribution reminiscent of white pulp areas of adult spleen. Although our results indicate that LTi cells do not directly contribute to white pulp development, the localization of these cells, their close association with blood vessels, their probable interaction with VCAM-1+ICAM-1-•/lo stromal cells at E18.5 is intriguing, but might just be a reflection of the chemokines and adhesion molecules that are locally expressed.

The Cxcl12 and Cxcl13 transcripts were similarly expressed within the ‘stromal’ and ‘endothelial’ stromal cell subset. On the contrary, the CD34+ICAM+ ‘endothelial’ stromal cell subset expressed higher levels of Ccl19 and Ccl21b transcripts than the ‘stromal’ subset. Assuming that these relative transcript expressions are revealing the protein expression levels, we propose that the peri-vascular distribution of LTi cells could be mediated by the high expression of CCL19 and CCL21 chemokines from the endothelial cells. Indeed, the lower expression of CCL19 and CCL21 by the stromal...
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Figure 4: Early priming of the fetal spleen stroma. LTi cells maintain in fetal spleen graft and co-localize with B cells. (A) Three weeks after engraftment of CD45.2+ fetal spleens under the kidney capsule of CD45.1+ Rag2/yc-/- mice, grafts were analyzed by immunohistology for LTi cells (CD4 in green), B cells (B220 in white) and erythrocytes (Ter119 in red) (FS graft). The top panel represents LTi cells forming a ring like structures (white ovals) with a vein (arrowhead) devoid of LTi cells. The middle panel displays LTi cells and B cells in close contact to each other. The same experiment supplemented with injection of CD45.2+ fetal liver cells was used as a positive control for splenic architecture and most CD4+ cells (green) are T cells (FS graft + FL injection). Blue bar: 200 µm. (B) Grafts were analyzed for their composition. Cells were separated between graft-derived (CD45.2+) and host-derived (CD45.1+) and analyzed by flow cytometry for the presence of LTi (CD4+CD3εIl7r+), B (CD19+B220+ and B220+CD21+), NK (NK1.1+CD3ε-), DC (CD11c+) and T cells (CD3ε+). Data are representative for analysis of three individual grafted mice.
Figure 5: Lymphotoxin-β R binding cells in neonatal spleen and MLN. LTβR-human IgG binding to B cells (A, B) or LTi cells (C, D) was analyzed by flow cytometry at indicated days after birth and 4 mice per group were analyzed.
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Figure 6: Early stages of white pulp development occur in the absence of LTα. Immunofluorescence staining of 2, 4 and 6 day-old spleens from C57BL/6 (B6) and LTα−/− mice were done using (A) anti-B220, anti-CD3 and anti-MOMA-2 antibodies to detect respectively B cells, T cells and macrophages and (B) anti-VE-Cadherin, anti ICAM-1, and anti VCAM-1 antibodies to detect stromal and endothelial cell subsets. Data are representative of three individual mice per group.
cells would establish a CCL19 and CCL21 concentration gradients decreasing from the vascular endothelial cells to the inner part of the fetal spleen. The CXCL12 and CXCL13 chemokine expressions by the splenic stroma probably also participate in the attraction and retention of LTi cells into the fetal spleen. Early expression of CXCL13, before the entry of B cells, has been observed around arterioles in developing human spleen \(^{27}\). Thus, expression of these homeostatic chemokines most likely contributes to the periarteriolar localization of LTi cells in the fetal spleen.

Our graft experiments show LTi and B cells co-localizing into well-defined areas within fetal spleen, illustrating the early specific capacities of the spleen stromal cells. In the grafts, all B and LTi cells are donor-derived and have differentiated \textit{in situ}. Both spleen stromal populations expressed the transcripts for CXCL13 and CXCL12, important for LTi cell interaction and B cell attraction and differentiation \(^{11, 43, 44}\). Moreover, we observed a small host-derived CD11c\(^+\) dendritic cell population in these grafts. It was shown that CD11c\(^+\) cells can be found in a neonatal spleen only a few days after birth \(^{45, 46}\). Hence, dendritic cells have migrated into the grafted spleen, in response to the chemokines produced.

In our grafts a typical adult spleen organization was observed when total fetal liver cells were previously injected. The T and B lymphocytes that differentiated from these fetal liver cells, were attracted by the fetal spleen graft into their specific domains reinforcing the capacity of the white pulp anlagen to express chemokines essential for the development of its’ architecture. It was shown that CCL21 could be expressed by two different genes after an activation of either a LT\(\alpha_1\beta_2\) dependent or independent pathway whereas the two chemokines conserve the same chemotactic activity \(^{47}\). Most of CCL21 expression depends on LT\(\alpha_1\beta_2\) in the adult spleen but not in non-lymphoid tissue \(^{47}\). It is possible that the first chemokine expression found in the fetal spleen at E15.5 is triggered in a similar LT\(\beta R\) independent manner.

Finally, our data show that the fetal spleen stroma is instructed at an early timepoint to segregate lymphoid and non-lymphoid zones, which correlates with red and white pulp areas of the adult organ. The graft experiments and the low numbers of B or LTi cells in the fetal spleen that express the LT\(\beta R\) suggest that these first driving events are LT\(\beta R\) independent.
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Supplementary Figure 1: Murine fetal spleens at developmental stages E18.5, E16.5 and E14.5. Immunofluorescence staining of sections from E18.5 (A-H), E16.5 (I-K) and E14.5 (L-O) spleens. Merge image (C) of double staining for MAdCAM-1 and VE-cadherin showing double labeled vascular endothelial cells. (D) Overview image of FS containing LTi cells characterized by their expression of RORγt, Il7r and CD4. Higher magnification images show a cluster of LTi cells expressing RORγt (E), Il7r (F) and CD4 (G). (H) Merge image of (E-G). FS of developmental stage E16.5 expressing MAdCAM-1 (I) and VE-cadherin (J). (K) Merge image of I and J. Double arrowhead indicates a large vessel with low MAdCAM-1 expression in the center of E14.5 FS. (L) VCAM-1 expression is abundant in the stomach (double arrowhead) but nearly absent in the spleen (arrowhead). In contrast, ICAM-1 and MAdCAM-1 are expressed in the FS at this developmental stage, but are absent in the stomach. (O) Merge image of L-N, showing ICAM-1⁺MAdCAM-1⁺ cells in the FS and VCAM-1⁺ cells in the stomach.
Supplementary Figure 2: CD4hiLin- cells from the E15.5 fetal spleen express the RORγ/γ(t) transcripts β-actin and RORγ/γ(t) transcript expression from E15.5 FS CD4hiLin- cells and 3 days old intestine CD4+I7r+CD3ε- cells.
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**Supplementary Figure 3:** Control staining for aspecific binding of LTβR-human IgG in splenic versus MLN lymphoid populations. LTβR-human IgG was incubated together with blocking anti LTβ antibody (BBF6), which was shown before to prevent binding of LTβR-human-IgG to surface expressed LTαβ, [8]. Aspecific binding of LTβR-human IgG to LTi cells (A) was analyzed by flow cytometry at indicated days after birth and 4 mice per group were analyzed. B. Lymphotoxin-βR-human IgG binding expressing LTi cells (staining) corrected for aspecific binding (block) as percentage of total LTi cells. A significant part of MLN derived LTi cells bind LTβR-human IgG, while LTβR-human IgG binding LTi cells are nearly absent in neonatal spleens (staining - block).
Supplementary Fig. 4. Murine splenic white pulp at day 6. Confocal images of B220, CD3, and MOMA-2 showing distinct locations of B and T cells (A,B) and of macrophages (C). D. Merge image showing macrophages that are present in B cell areas (i), T cell areas (ii) and red pulp (iii). Macrophages are also present in the vicinity of the central arteriole (arrowhead). VE-cadherin (E), ICAM-1 (F) and VCAM-1 (G) expression by stromal and endothelial cells. H. Stromal cells in the splenic white pulp are characterized by a VCAM-1*ICAM-1+ (i) around the central arteriole (arrowhead). At the periphery of this area VCAM-1*ICAM-1+ (ii) stromal cells are present.